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Is Required for Proper Differentiation of Mouse Gastric Epithelium

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During mouse embryogenesis GATA-4 is expressed first in primitive endoderm and then in definitive endoderm derivatives, including glandular stomach and intestine. To explore the role of GATA-4 in specification of definitive gastric endoderm, we generated chimeric mice by introducing *Gata4*$^{-/-}$ ES cells into *ROSA26* morulae or blastocysts. In E14.5 chimeras, *Gata4*$^{-/-}$ cells were represented in endoderm lining the proximal and distal stomach. These cells expressed early cytodifferentiation markers, including GATA-6 and ApoJ. However, by E18.5, only rare patches of *Gata4*$^{-/-}$ epithelium were evident in the distal stomach. This heterotypic epithelium had a squamous morphology and did not express markers associated with differentiation of gastric epithelial cell lineages. Sonic Hedgehog, an endoderm-derived signaling molecule normally down-regulated in the distal stomach, was overexpressed in *Gata4*$^{-/-}$ cells. We conclude that GATA-4-deficient cells have an intrinsic defect in their ability to differentiate. Similarities in the phenotypes of *Gata4*$^{-/-}$ chimeras and mice with other genetically engineered mutations that affect gut development suggest that GATA-4 may be involved in the gastric epithelial response to members of the TGF-β superfamily.

Key Words: **endoderm; stomach; transcription factor; H^+, K^+-ATPase; sonic hedgehog.**

INTRODUCTION

Development of the stomach, like other gastrointestinal organs, entails several sequential steps: differentiation of definitive endoderm, formation and regional specialization of the primitive gut tube, followed by morphogenesis and differentiation of component cell lineages (Grapin-Botton and Melton, 2000). Insights into the factors that regulate gut development have been gleaned from model organisms (Wells and Melton, 1999; Yasuo and Lemaire, 1999). Tissue recombination experiments and gene targeting studies have shown that differentiation of gastric epithelium is controlled by mesenchyme-derived signals, including bone morphogenetic proteins (BMPs) and other members of the transforming growth factor-β (TGF-β) superfamily (Hayashi *et al.*, 1988; Kaestner *et al.*, 1997; Kim *et al.*, 2000; Koike and Yasugi, 1999; Narita *et al.*, 2000; Smith *et al.*, 2000; Tabata and Yasugi, 1998). Endoderm-derived signaling molecules, such as Sonic hedgehog (Shh) and Indian hedgehog (Ihh), also play a role in stomach morphogenesis (Ramalho-Santos *et al.*, 2000; Sukegawa *et al.*, 2000). However, the transcription factors required for specification and differentiation of the principal gastric epithelial cell lineages remain poorly characterized.

In the adult mouse, the proximal third of the stomach (fore-stomach) is lined by a squamous epithelium, while the distal two-thirds is covered by a glandular epithlium containing thousands of mucosal invaginations known as gastric units (Karam *et al.*, 1997; Karam, 1998; Nomura *et al.*, 1998). Each unit in the central portion of the stomach contains approximately 200 cells representing the three principal lineages. The apical third of the unit, the pit region, is populated with mucus-producing cells. A centrally positioned isthmus contains the multipotent stem cell(s) and their undifferentiated yet committed descen-

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dants. The neck region is located just below the isthmus and contains mucus-producing neck cells. The base of the gastric unit contains pepsinogen-positive zymogenic cells. Acid-producing parietal cells are found in all four regions of the gastric unit. One of the stem cell's committed daughters, the granule-free pit cell precursor, gives rise to prepit cells within the isthmus which become pit cells as they migrate up the pit. Zymogenic cells differentiate during a downward migration from the isthmus to the base of the gastric units, during which time they undergo a series of morphologic transformations (granule-free preneck cell precursor to preneck cells to neck cells to prezymogenic and finally zymogenic cells). Parietal cells complete their differentiation within the isthmus (granule-free preparietal to preparietal to mature parietal cell), after which time they undergo bipolar migration to both the pit and base.

Gastric unit morphogenesis begins during late fetal life, when the endoderm undergoes cytodifferentiation to an epithelial monolayer with numerous short infoldings, termed "primordial buds" (Karam *et al.*, 1997). Approximately 10% of the epithelial cells in these primordial buds have the morphologic features of differentiated pit, neck, parietal, or enteroendocrine cells. Most of the remaining cells resemble granule-free lineage progenitors. From postnatal day 1 (P1) to P7, the total number of cells per bud does not change, but the fractional representation of differentiated pit and neck cells increases. Between P7 and P15, the buds elongate and form nascent gastric units. Distinct pit, isthmus, neck, and base compartments are evident by P28. Stomach glands commence development as polyclonal units, but by 6 weeks of age the majority have progressed to monoclonal units (Nomura *et al.*, 1998).

GATA transcription factors have emerged as critical regulators of endoderm differentiation and development in a variety of organisms (Bielinska *et al.*, 1999; Crispino *et al.*, 2001; Fukushige *et al.*, 1999; Ghatpande *et al.*, 2000; Kostetskii *et al.*, 1999; Molkentin, 2000; Paré *et al.*, 2001; Rehorn *et al.*, 1996; Reiter *et al.*, 1999, 2001; Sakamoto *et al.*, 2000; Shoichet *et al.*, 2000; Weber *et al.*, 2000; Zaret, 1999; Zhu *et al.*, 1997). *In vivo* footprinting studies suggest that GATA-binding proteins, in conjunction with other transcription factors such as HNF-3/fork head factors, impart genes with the competence to be selectively activated in endodermal derivatives (Bossard and Zaret, 1998, 2000; Denson *et al.*, 2000). *Trans*-activation and electrophoretic mobility shift assays indicate that GATA-4, -5, and -6 regulate genes known to be expressed in parietal cells [H^+/K^+-*ATPase-β subunit* (Mushiake *et al.*, 1994; Nishi *et al.*, 1997b; Tamura *et al.*, 1993)], pit cells [*trefoil factors 1* and *2* (Al-azzeh *et al.*, 2000)], and neck/zymogenic cells [*pepsinogen* (Sakamoto *et al.*, 1998, 2000)].

Mice deficient in GATA-4, -5, or -6 have been generated and their phenotypes described. *Gata4*$^{-/-}$ mice die by E9.5 and exhibit severe defects in ventral morphogenesis and heart tube fusion (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). In these embryos, definitive endoderm forms, but the gut tube does not close. Chimera analysis has shown that wild-type endoderm, presumably visceral endoderm, can abrogate the ventral developmental defects associated with GATA-4 deficiency (Narita *et al.*, 1997b). *Gata6*$^{-/-}$ mice die

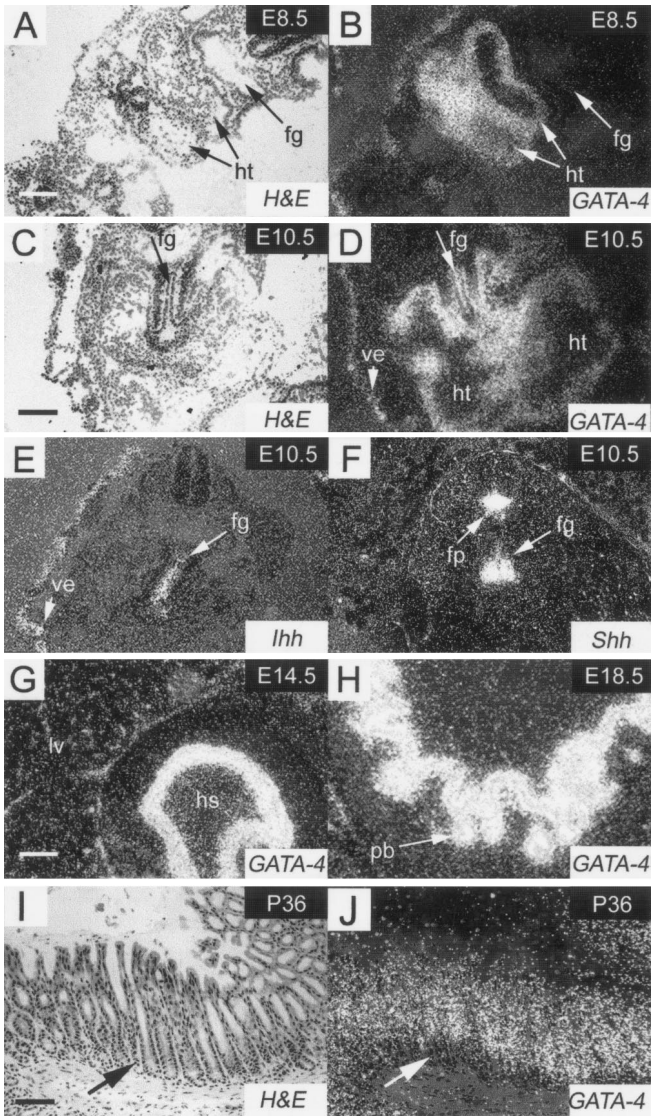


FIG. 1. *Gata4* mRNA expression in embryonic, fetal, and adult mouse gut. Tissue sections were prepared from E8.5 (A, B), E10.5 (C–F), E14.5 (G), E18.5 (H), or P36 (I, J) mice and subjected to *in situ* hybridization to detect mRNAs encoding GATA-4 (B, D, G, H, J), Ihh (E), or Shh (F). Bright field (A, C, I) and dark field (B, D–H, J) views are shown. At E8.5 and E10.5, GATA-4 mRNA is not evident in the rostal foregut, but is present in endoderm located at the foregut–midgut junction (A–D). The expression of GATA-4 overlaps with that of Ihh (E) and partially overlaps with that of Shh (F). At E14.5, GATA-4 mRNA is present in the columnar epithelium of the hind-stomach, but not the fore-stomach (G). At E18.5, GATA-4 mRNA is evident in the primordial buds of the glandular gastric epithelium (H). In adult animals, GATA-4 mRNA is expressed in the pit, neck, and isthmal regions of gastric units, but not in the base (I, J; arrow). Abbreviations: fg, foregut; fp, floorplate; hs, hind-stomach; ht, heart; lv, liver; pb, primordial buds; ve, visceral endoderm. Bars, 100 μm.

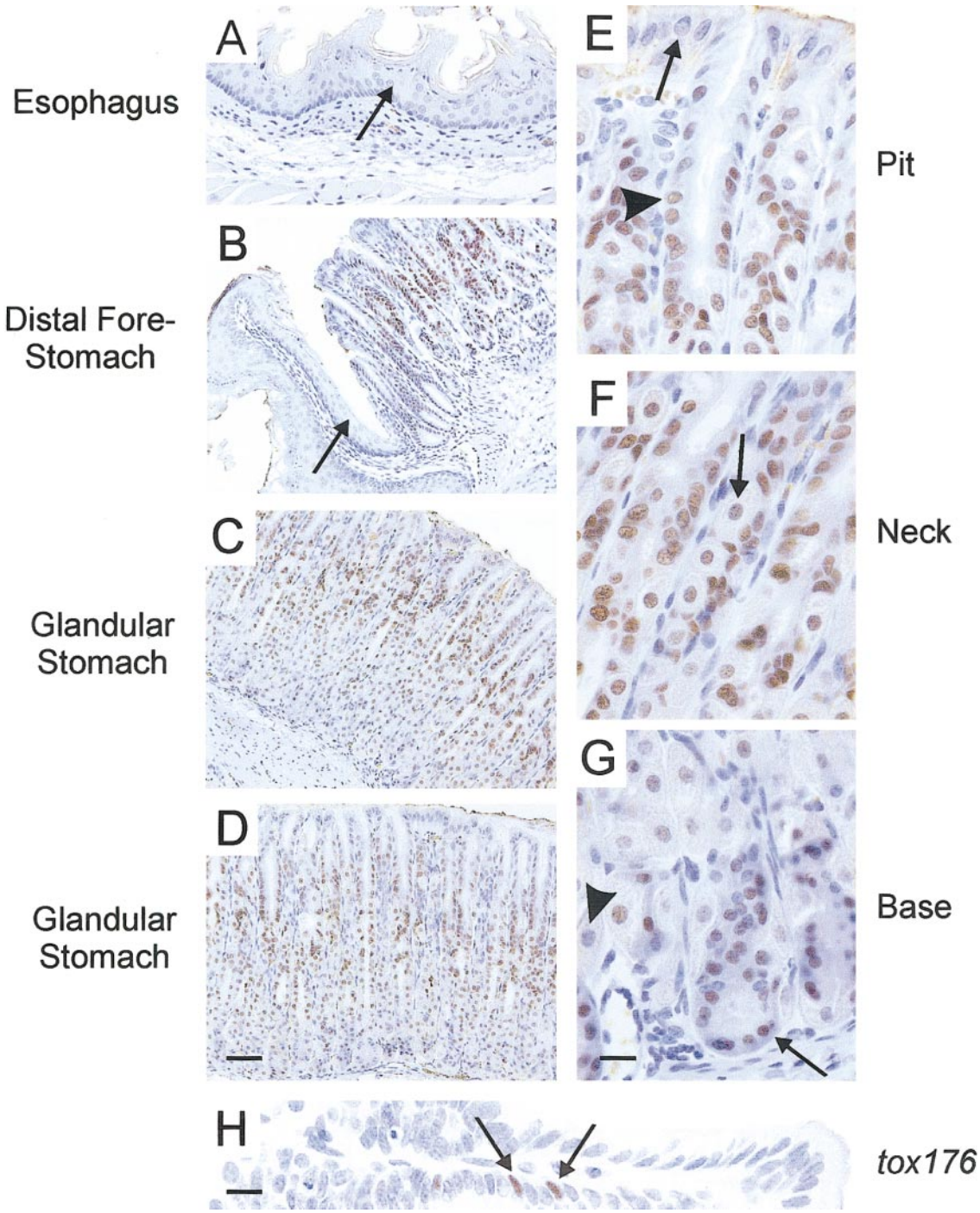
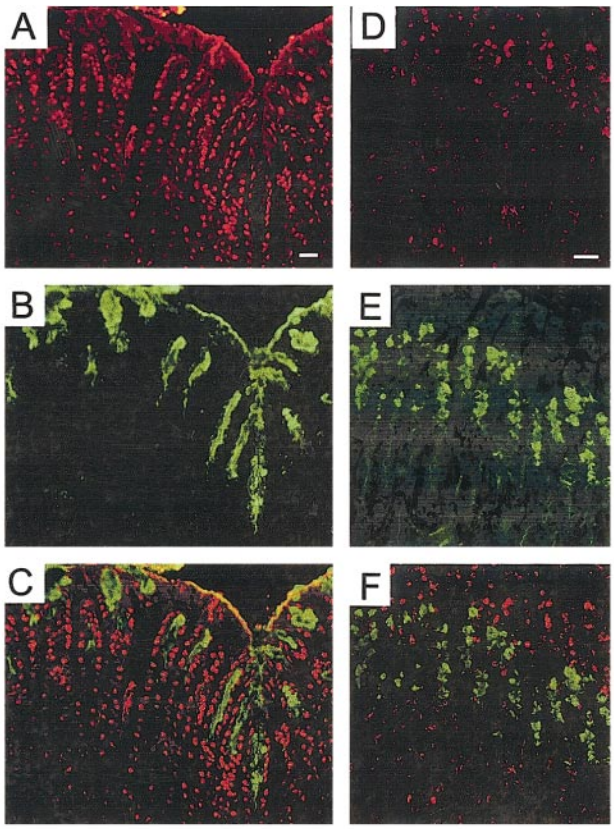


FIG. 2. GATA-4 protein expression in foregut derivatives of the adult mouse. Formalin-fixed, paraffin-embedded issue sections from a 6-week-old normal mouse or H^+/K^+-*ATPase β-subunit* $^{-1035 \rightarrow +24}$ promoter-*tox176* mouse were subjected to immunoperoxidase staining for GATA-4 and then counterstained with hematoxylin. The nuclei of cells expressing GATA-4 stain brown, whereas nonexpressing cells stain blue. (A) Squamous epithelial cells in the esophagus (arrow) do not express GATA-4. (B) Squamous epithelial cells of the fore-stomach (arrow) do not express GATA-4, but cells in the adjoining glandular gastric epithelium do. (C, D) GATA-4 staining is most intense in epithelial cells within the central portion of the gastric unit. (E) On higher magnification, GATA-4 expression is evident in mucus-secreting pit cells. Those deeper in the gland (arrowhead) express more GATA-4 than cells at the apical surface (arrow). A mitotic figure near the bottom of the panel indicates a region of active cell proliferation (isthmus). (F) Parietal cells (arrow) and zymogenic cell precursors in the neck region express GATA-4. (G) In the base of the gastric unit, weak GATA-4 expression is evident in some zymogenic cells (arrow) and in some, but not all parietal cells (arrowhead). (H) *tox176* mice show reduced GATA-4 expression reflecting the lack of parietal and preparietal cells. The residual GATA-4-positive cells (arrows) may represent progenitors that give rise to pit cells and other lineages. Bars, 100 μm (D), 30 μm (G), 30 μm (H).



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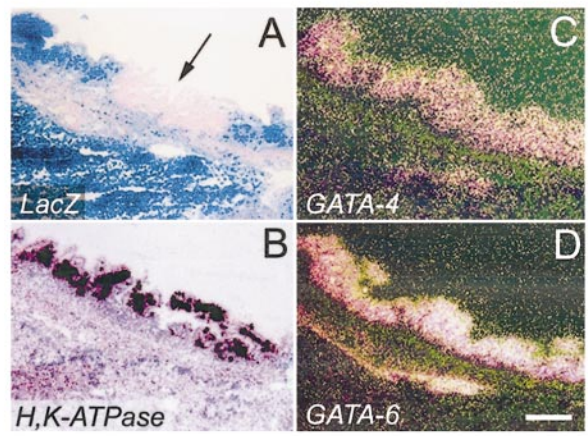

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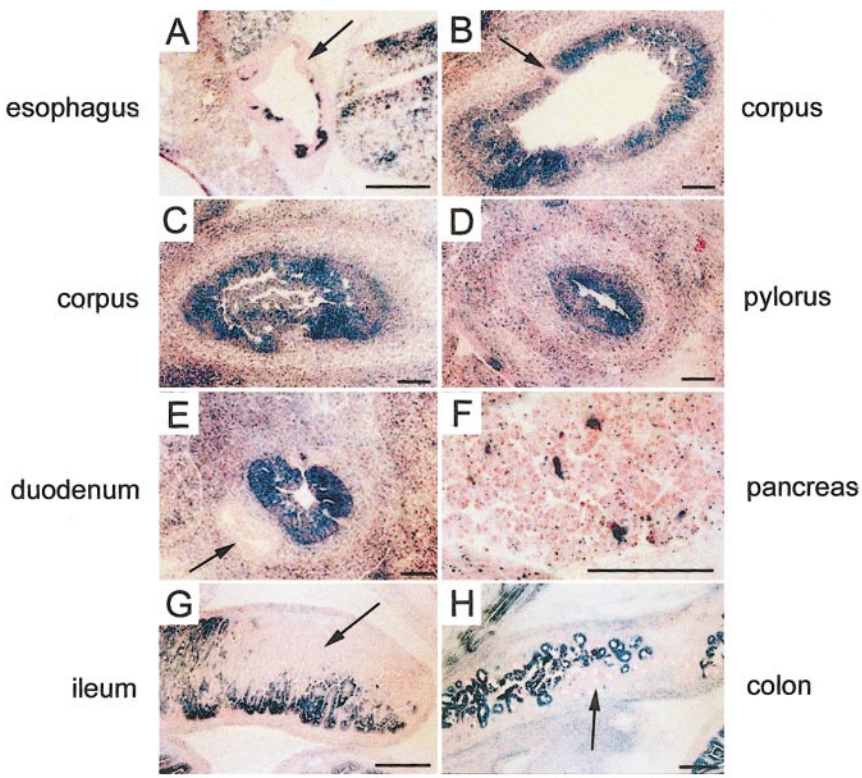

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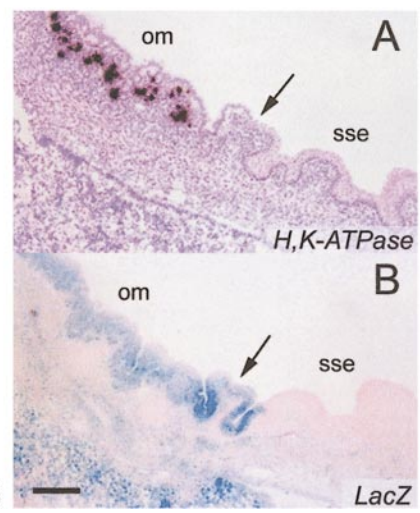

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in the early postimplantation period from defects in extraembryonic endoderm (Koutsourakis *et al.*, 1999; Morrisey *et al.*, 1998, 2000). Chimera studies have shown that *Gata6*$^{-/-}$ ES cells are impaired in their ability to differentiate into bronchial epithelium (Morrisey *et al.*, 1998). Targeted mutagenesis of *Gata5* results in female genitourinary tract abnormalities but no overt defects in endoderm development or function (Molkentin *et al.*, 2000).

In this study, we have utilized chimeric mice to examine the role of GATA-4 in gastric epithelial morphogenesis. We show that GATA-4 deficiency is associated with impaired differentiation into glandular but not squamous epithelium. Similarities between the phenotypes of *Gata4*$^{-/-}$ chimeras and other gut development mutants suggest that GATA-4 may function in concert with members of the TGF-β superfamily to promote gastric epithelial specification/differentiation.

METHODS

Animals. *ROSA26* mice, bearing a ubiquitously expressed β-galactosidase transgene on a strain *129* background (Friedrich and Soriano, 1992), were obtained from Jackson Labs. FVB/N mice containing a transgene consisting of nucleotides −1035 to +24 of the β-subunit gene of mouse *H*$^+$*/K*$^+$*-ATPase* linked to an attenuated diptheria toxin A fragment (*tox176*) have been described earlier (Li *et al.*, 1996; Syder *et al.*, 1999).

Generation and analysis of chimeric mice. *Gata4*$^{-/-}$*/ES* cells were prepared and maintained in culture as described previously (Kuo *et al.*, 1997; Soudais *et al.*, 1995). To generate the chimeric embryos, male mice homozygous for the *ROSA26* transgene (Friedrich and Soriano, 1991) were mated to supraovulated C57BL/6-J females. Eight-cell stage *ROSA26* embryos were harvested at 2.5 d.p.c. and each morula was injected (Hogan *et al.*, 1994) with 1–4 *Gata4*$^{-/-}$ ES cells. Alternatively, C57Bl/6 blastocysts were injected with lacZ-tagged *Gata4*$^{-/-}$ ES cells (Kuo *et al.*, 1997). Injected embryos were transferred to pseudopregnant Swiss–Webster females.

X-Gal staining of whole-mount embryos and tissue sections. Chimeras were initially identified by PCR or GPI isoenzyme analysis (Papaioannou and Johnson, 1993). PCR of lacZ sequences was performed by using tail DNA, 5′-TGGCATGTGAGGCGGTTAGGTTATCT-3′ and 5′-GAGCTTTGCCACATCACAGGTCATTC-3′ as forward and reverse primers, respectively, and 25–35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. To visualize LacZ expression in late gestation animals, frozen sections (10 μm) were prepared by embedding animals in OCT cryopreservation solution (Tissue-Tek). Sections were then fixed with 0.2% gluteraldehyde for 5–15 min, permeabilized with 100 mM potassium phosphate, pH 7.4, 0.02% NP-40, and 0.01% sodium deoxycholate for 5–15 min, and then incubated in 0.5 mg/ml X-gal (Promega) with 10 mM $K_3[Fe(CN)_6]$, 10 mM $K_4[Fe(CN)_6]$, 100 mM potassium phosphate, pH 7.4, 0.02% NP-40, and 0.01% sodium deoxycholate at 37°C overnight. Sections were counterstained with eosin and examined under bright and dark field (Narita *et al.*, 1997a).

In situ hybridization. Frozen sections (10 μm) were fixed with 4% paraformaldehyde in PBS and subjected to *in situ* hybridization as described (Heikinheimo *et al.*, 1994). Sections were incubated with 10^6 cpm of [^{33}P]-labeled antisense riboprobe prepared using the following linearized plasmid templates and RNA polymerases: (i), GATA-4, *Bam*HI-digested pBluescript SK phagemid λG14a (Arceci *et al.*, 1993), T7 polymerase; (ii) GATA-6, *Eco*RV-digested pCRII subclone, Sp6 polymerase (Narita *et al.*, 1996); (iii) H$^+$,K$^+$-ATPase-β, *Hin*dIII-digested pBluescript SK II$^+$ plasmid containing the cDNA, T3 polymerase (Ketola *et al.*, 1999); (iv) Ihh, *Xba*I-digested pBluescript SK plasmid containing the cDNA, T7 polymerase (Bitgood and McMahon, 1995); (v) Shh, *Hin*dIII-digested pBluescript SK plasmid containing the cDNA, T3 polymerase (Bitgood and McMahon, 1995); (vi) ApoJ, *Bam*HI-digested pBluescript SK plasmid containing the cDNA (Aronow *et al.*, 1993).

Single and multilabel immunohistochemical studies. Frozen sections (10 μm) were fixed with 4% paraformaldehyde and processed for immunohistochemistry as described (Narita *et al.*, 1997a). Alternatively, tissue was fixed with 10% formalin in PBS, embedded in paraffin, sectioned (4 μm), and then processed for

FIG. 3. Lectin and antibody staining of gastric units in the corpus region of adult mice. Formalin-fixed paraffin sections from 6-week-old FVB/N mice were subjected to multilabel fluorescent staining. GATA-4 protein was detected by using goat anti-GATA-4 antibody and rhodamine-conjugated rabbit anti-goat IgG (A, D). FITC-labeled UAE-I lectin was used to stain pit and mucus cells (B), and FITC-labeled GSII lectin was used to stain neck cells (E). Panel (C) is a composite of (A) and (B), while Panel (F) is a composite of (D) and (E). Most of the GATA-4 staining is in the central region of the gastric unit. Bars, 25 μm.

FIG. 4. Wild-type ES cells contribute to glandular gastric epithelium in E17.5 chimeric embryos. The stomach lumen is at the top of each panel, and liver is at the bottom. X-Gal staining discriminates ES cell-derived epithelium (arrow) from host-derived (blue) epithelium (A, bright field). Parallel tissue sections were subjected to *in situ* hybridization for H$^+$/K$^+$-ATPase β-subunit (B, bright field), *GATA-4* (C, dark field), or *GATA-6* (D, dark field). Note that wild-type ES cells differentiate into glandular gastric epithelium that expresses each of these markers in the correct temporospatial manner. Bar, 100 μm.

FIG. 5. *Gata4*$^{-/-}$ ES cell descendants are rare in glandular gastric epithelium of E16.5 *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimeras. Panoramic view of the distribution of *Gata4*$^{-/-}$ cells along the length of the gastrointestinal tract of an E16.5 chimera. Sections were genotyped with X-gal/eosin. GATA-4-deficient cells contribute readily to the esophagus (A, arrow), duodenum (E, arrow), pancreatic acinar cells (F), ileum (G, arrow), and colon (H, arrow). In contrast, *Gata4*$^{-/-}$ cells are rare in the glandular stomach epithelium (e.g., the arrow points to a small stripe of GATA-4-deficient epithelium in (B). Bar, 0.5 mm.

FIG. 6. Transition zone between the fore-stomach and hind-stomach in an E18.5 *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimera. Adjacent tissue sections were subjected to (A) *in situ* hybridization for H$^+$/K$^+$-ATPase β-subunit, or (B) X-gal staining. The point of transition between oxyntic mucosa (om) and stratified squamous epithelium (sse) of the fore-stomach is indicated by the arrow. *Gata4*$^{-/-}$ ES cells contribute to the squamous epithelium of the fore-stomach but not glandular gastric epithelium. Bar, 100 μm.

immunohistochemistry (Ketola *et al.*, 1999; Li *et al.*, 1995). Sections were stained with the following primary antibodies: (i) rabbit anti-pepsingogen [final dilution in blocking buffer (1% bovine serum albumin, 0.3% Triton X-100, 1 mM $CaCl_2$ in PBS = 1:200); specificity = zymogenic cells (Lorenz and Gordon, 1993)]; (ii) goat anti-GATA-4 (dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA); or (iii) affinity purified rabbit anti-GST-GATA-4 4 (dilution 1:100) (Narita *et al.*, 1997a); mouse anti-pancytokeratin (dilution 1:50, blocked with mouse IgG F(ab) fragment 1:100, Sigma, St. Louis, MO); goat anti-E-cadherin (dilution 1:100, Sigma).

Antigen-antibody complexes were detected with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA), biotin-conjugated goat anti-mouse antibody (1:200, Vector Laboratories, Burlingame, CA) or biotin-conjugated donkey anti-goat antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and the avidin-biotin immunoperoxidase system (Santa Cruz Biotechnology). Alternatively, complexes were visualized with tyramine signal amplification using reagents and protocols supplied by NEN Life Science Products (Boston, MA) or rhodamine conjugated rabbit anti-goat antibody [1:1000; Chemicon, Temecula,CA (Narita *et al.*, 1997a)]. Slides were then counterstained with hematoxylin and/or eosin.

Lectin staining was performed on formalin-fixed tissue sections. The lectins used were FITC-labeled *Ulex europaeus* agglutinin I [UAE-I; Sigma; 1:200; cellular specificity = pit and surface mucus cells (Falk *et al.*, 1994)] and FITC-labeled *Griffonia simplifolica* II [GSII; Sigma; 1:200; cellular specificity = neck cells (Falk *et al.*, 1994; Nomura *et al.*, 1998)]. Lectins were diluted to a final concentration in PBS blocking buffer. The methods used for multilabel staining of sections are described elsewhere (Falk *et al.*, 1994).

Frozen sections (10 μm) were fixed with 4% paraformaldehyde for periodic acid Schiff (PAS) staining using a commercially available kit (Sigma). Stained sections were photographed with an Olympus AHB2 microscope and Kodacolor 400 film.

RESULTS

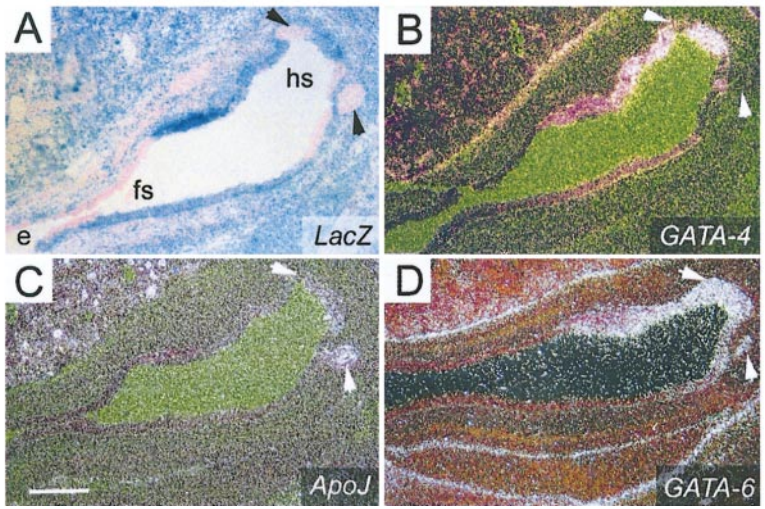
Expression of GATA-4 in the stomach of normal and transgenic mice. While expression of GATA-4 in mouse extraembryonic endoderm, cardiomyocytes, intestinal enterocytes, adrenal, and gonadal cells has been carefully delineated (Heikinheimo *et al.*, 1994; Ip *et al.*, 1994; Ketola *et al.*, 1999; Kiiveri *et al.*, 1999; Morrisey *et al.*, 1996; Viger *et al.*, 1998), relatively little is known about GATA-4 expression in the developing stomach (Mushiake *et al.*, 1994). To gain a more complete picture of the role of this transcription factor in gastric morphogenesis, we examined the expression pattern of GATA-4 in the developing stomach of normal mice, using a combination of *in situ* hybridization and immunohistochemistry.

GATA-4 mRNA was evident in definitive endoderm cells near the foregut/midgut junction of embryonic day 8.5–10.5 mice, but not in more rostal foregut endoderm (Figs. 1A–1D). This pattern of expression overlapped with that of Ihh, an endodermal signaling molecule implicated in foregut development (Fig. 1E) (Bitgood and McMahon, 1995; Ramalho-Santos *et al.*, 2000). The related signaling molecule, Shh, was expressed in a subset of GATA-4-positive foregut endoderm cells (Fig. 1F). At E14.5, GATA-4 mRNA was evident in the nascent epithelium of the distal stomach (Fig. 1G) but not the fore-stomach (data not shown). By E18.5, GATA-4 mRNA was detected in primordial buds of the glandular gastric epithelium (Fig. 1H). Abundant GATA-4 mRNA was observed in the glandular gastric epithelium of the adult mouse (Figs. 1I and 1J). Within gastric units of the adult stomach, GATA-4 mRNA was more prominent in the neck, isthmus, and pit regions than in the base (Figs. 1I and 1J, arrows).

Immunohistochemistry and lectin staining were used to further delineate the pattern of GATA-4 expression in the adult mouse (Figs. 2 and 3). GATA-4 protein was not seen in squamous epithelial cells of the esophagus (Fig. 2A) or fore-stomach (Fig. 2B), but abundant GATA-4 was detected in nuclei of epithelial cells within the glandular stomach (Figs. 2C–2G). Although GATA-4 protein was evident in all regions of the gastric unit (pit, isthmus, neck, and base), staining was most intense in the central portion of each unit (Figs. 2C, 2D, 3A, 3C, 3D, and 3F). Pit cells near the isthmus had more GATA-4 protein than those near the apical surface (Figs. 2E, 3A–3C), suggesting that expression this factor is down-regulated during pit cell migration up the gland. Most parietal cells in the pit and neck (Fig. 2F) regions expressed GATA-4 protein, whereas only a subset of parietal cells in the base expressed this protein (Fig. 2G). Faint GATA-4 staining was seen in zymogenic cells in the base of the glands (Fig. 2G).

To determine whether GATA-4 is expressed in isthmal lineage progenitors, we surveyed adult *tox176* mice (Li *et al.*, 1996; Syder *et al.*, 1999). These mice contain a transgene consisting of nucleotides $-1035 \rightarrow +24$ of the *H^+/K^+-ATPase β-subunit* gene linked to an attenuated diphtheria toxin A fragment. The transcriptional regulatory elements from the β-subunit gene are only active in preparietal cells and their differentiated descendants (Li *et al.*, 1996). Parietal cells are completely eliminated from all gastric units in the pedigree of *tox176* mice studied (Syder *et al.*, 1999). Loss of parietal cells is accompanied by inhibition of the terminal differentiation of neck to zymogenic cells, suggesting that parietal cells are a source of the factors necessary for terminal differentiation of this lineage. Parietal cell ablation in *tox176* mice is accompanied by enhanced proliferation of the presumptive multipotent stem cell and its committed granule-free daughters, which become a predominant population between 4 and 16 weeks of age. As noted in Introduction, parietal cells are the only principal gastric epithelial cell types that complete their differentiation within the isthmus. The expansion of progenitor cells in response to parietal cell ablation suggests that the strategically placed isthmal parietal cell is the source of factors that affect the proliferative status neighboring isthmal lineage progenitors.

We observed reduced levels of GATA-4 expression in the glandular stomach of *tox176* mice (Fig. 2H). This result supports the notion that GATA-4 is expressed in preparietal cells and mature parietal cells of wild-type mice. Rare



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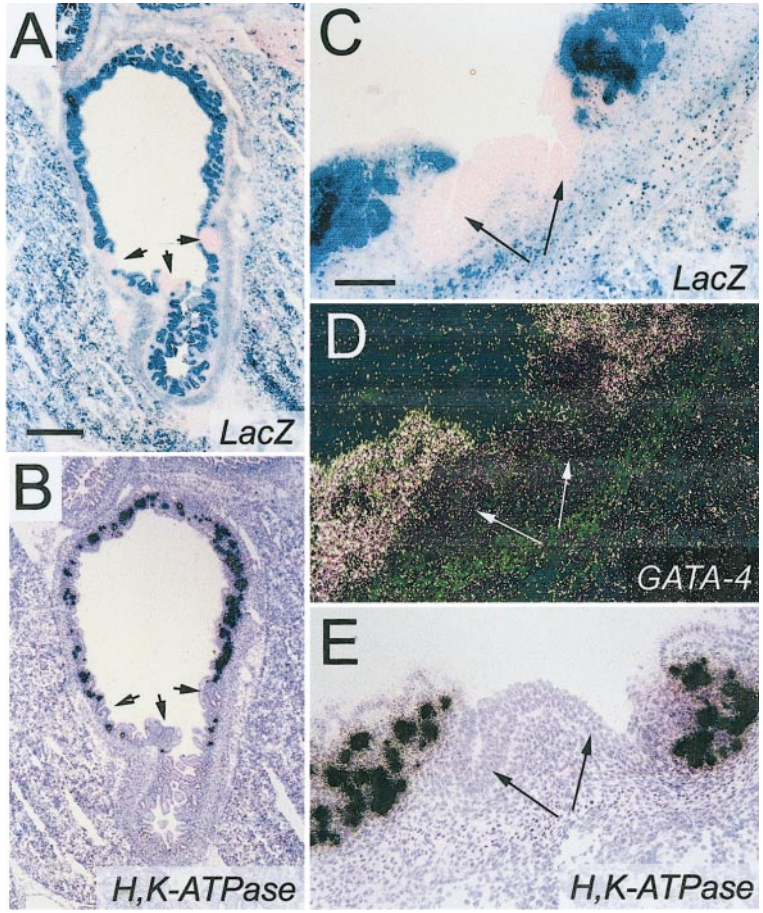

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FIG. 7. Contribution of *Gata4*$^{-/-}$ cells to E14.5 definitive fore-stomach and hind-stomach endoderm. Serial sections, prepared from an E14.5 *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimera, were genotyped with X-gal/eosin (A) and the subjected to *in situ* hybridization for GATA-4 (B), Apo-J (C), or GATA-6 (D). The arrows highlight patches of GATA-4-deficient epithelium. At this stage of development, levels of GATA-6 and Apo-J are comparable in juxtaposed patches of *Gata4*$^{+/+}$ and *Gata4*$^{-/-}$ endoderm in the distal stomach. Abbreviations: e, esophageal junction; fs, fore-stomach; hs, hind-stomach. Bar, 100 μm.

FIG. 8. Heterotypic GATA-4-deficient epithelium in the glandular stomach of E18.5 *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimeras. X-Gal and eosin staining distinguishes ES cell-derived epithelium (pink, arrows) from *Gata4*$^{+/+}$ (blue) epithelium (A, C, bright field). Adjacent sections were subjected to *in situ* hybridization for H^+/K^+-ATPase β-subunit mRNA (B and E, bright field) or GATA-4 mRNA (D, dark field). Note that *Gata4*$^{-/-}$ cells give rise to patches of atypical squamous epithelium that lack nascent gastric units (primordial buds) and lack cells that express a marker of the parietal cell lineage (i.e., H^+/K^+-ATPase β-subunit). Bars in A and B, 300 μm; C–E, 50 μm.

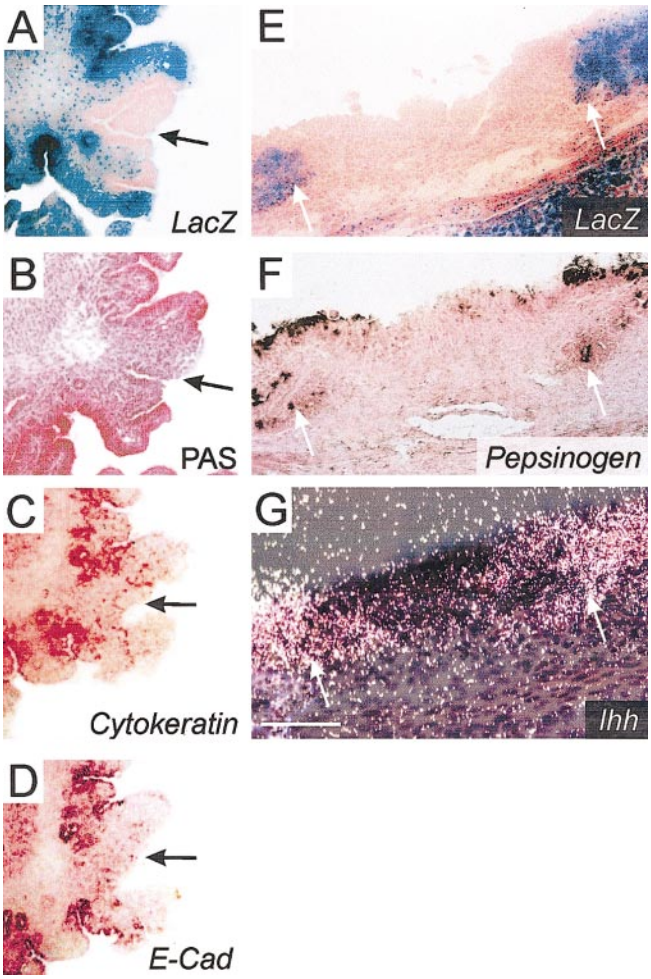

FIG. 9. Marker analysis of GATA-4-deficient glandular gastric epithelium in E18.5 *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimeric mice. Adjacent tissue sections were subjected to X-gal/eosin staining (A, E) or marker analysis. PAS staining was used to visualize glycol-containing cellular components (B). Cytokeratins were stained with mouse anti-pancytokeratin and biotinylated goat anti-mouse IgG (C). E-cadherin was detected by using goat anti-E-cadherin and biotinylated donkey anti-goat IgG (D). Pepsinogen was stained by using rabbit anti-pepsinogen and biotinylated donkey anti-rabbit IgG (F). Ihh mRNA was detected by *in situ* hybridization (G). Black arrows in (A–D) highlight GATA-4-deficient epithelium. White arrows in (E–G) point to *Gata4*$^{+/+}$ tissue. Note that *Gata4*$^{-/-}$ epithelium lacks expression of pit and zymogenic cell markers (PAS-positive mucin and pepsinogen, respectively), and the expression of other epithelial differentiation markers (cytokeratins, E-cadherin, Ihh) is dramatically reduced in this heterotypic epithelium. Bar, 100 μm.

GATA-4 expressing cells were evident in the gastric units of *tox176* mice (Fig. 2H, arrows). The location of these residual GATA-4 positive cells in the central portion of the gastric unit suggests that these cells may be progenitors that give rise to pit cells or other lineages.

Gata4*$^{-/-}$ *cells exhibit an intrinsic defect in gastric epithelial differentiation. GATA-4-null mice die before E9.5 (Kuo *et al.*, 1997; Molkentin *et al.*, 1997), so insights into the role of GATA-4 in stomach development cannot be ascertained using these animals. We used chimeric mice to circumvent this problem. *Gata4*$^{-/-}$ ES cells or control wild-type ES cells were injected into 8-cell stage embryos or blastocysts derived from *ROSA26* mice (Friedrich and Soriano, 1991), a transgenic line that expresses β-galactosidase in most cell types, including gut epithelial derivatives.

Control experiments using E9–E18.5 chimeras generated from wild-type ES cells established that ES cell-derived gastric epithelium does not produce a false-positive signal with X-gal staining (Fig. 4A). Furthermore, parietal cells present in primordial buds of E17.5 glandular gastric epithelium could readily be detected by *in situ* hybridization for H^+/K^+-ATPase β-subunit mRNA (Fig. 4B). Finally, we confirmed that wild-type ES cells contribute readily to glandular gastric mucosa and express GATA-4 and GATA-6 mRNAs at levels comparable to neighboring cells derived from *ROSA26* morulae (Figs. 4C and 4D).

E16.5–E18.5 *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimeras were then examined (n = 45). The contribution of GATA-4-deficient ES cells to various tissues was assessed in X-gal-stained serial sections. *Gata4*$^{-/-}$ cells contributed extensively to ectodermal and mesodermal derivatives, including the CNS, skin, heart, somites, limb buds, and

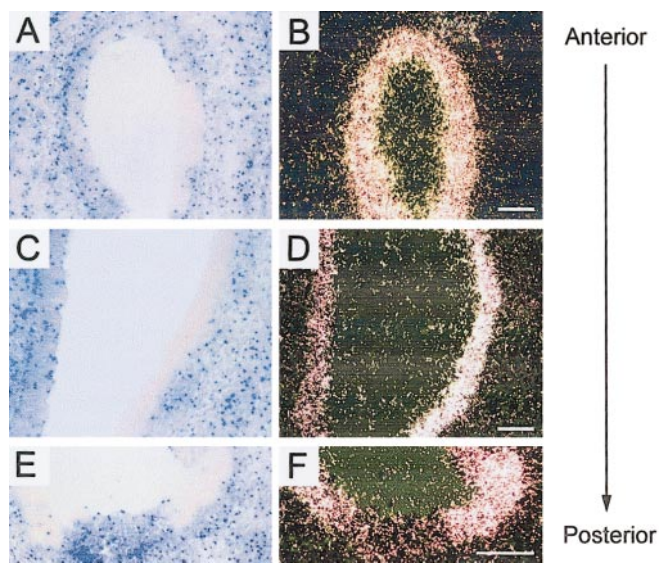

FIG. 10. Aberrant expression of *Shh* in *Gata4*$^{-/-}$ gastric epithelium of E14.5 *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimeric mice. Adjacent sections from the proximal (A, B), mid (C, D), or distal (E, F) stomach were subjected to X-gal/eosin staining or *in situ* hybridization for Shh. In the anterior stomach, Shh mRNA levels are equivalent in neighboring patches of *Gata4*$^{+/+}$ (blue) and *Gata4*$^{-/-}$ epithelium. In the mid and posterior stomach, Shh mRNA levels are relatively higher in *Gata4*$^{-/-}$ epithelium. Bars, 50 μm.

gut mesenchyme (data not shown). Moreover, *Gata4*$^{-/-}$ ES cell derivatives readily populated endoderm in the esophagus, fore-stomach, intestine, exocrine pancreas, and lung (Figs. 5 and 6). In contrast, the percentage of *Gata4*$^{-/-}$ cells represented in the E16.5–E18.5 gastric glandular epithelium was considerably lower than in esophageal, intestinal, or pancreatic epithelia (Figs. 5 and 6). This was true for chimeras derived from two independently selected *Gata4*$^{-/-}$ ES lines.

Next, we analyzed the expression of endodermal markers in E14.5–E18.5 *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimeras. In E14.5 mice, *Gata4*$^{-/-}$ cells contributed to endoderm derivatives in both the fore-stomach and hind-stomach (Figs. 7A and 7B). This hind-stomach GATA-4 deficient epithelium was morphologically indistinguishable from adjacent *Gata4*$^{+/+}$ endoderm. Both cell populations expressed two well-characterized markers of gut endodermal/epithelial differentiation: ApoJ (Aronow *et al.*, 1993) and GATA-6 (Morrisey *et al.*, 1996; Narita *et al.*, 1996) (Figs. 7C and 7D).

Though GATA-4-deficient epithelium in E14.5 chimeras resembled wild-type tissue, *Gata4*$^{-/-}$ epithelium in E17.5–E18.5 chimeras was grossly abnormal in appearance and marker expression. These cells had an atypical, squamous morphology (Figs. 8A, 8C, and 8D), similar to that reported in Type II activin receptor-deficient mice (Kim *et al.*, 2000). In contrast to neighboring *Gata4*$^{+/+}$ epithelium, the heterotypic GATA-4 deficient epithelium did not express markers associated with the parietal cell lineage (H^+/K^+-ATPase β-subunit mRNA; Figs. 8B and 8E). This abnormal epithelium did not stain with periodic acid-Schiff reagent, suggesting an absence of mucin-positive pit cells (Fig. 9B). There was little or no expression of cytokeratin (Fig. 9C) or E-cadherin (Fig. 9D) within the heterotypic epithelium. The GATA-4-deficient epithelium did not express pepsinogen, a zymogenic cell marker (Fig. 9F). Finally, the heterotypic epithelium lacked expression of Ihh (Fig. 9G). These results indicate that GATA-4 deficiency leads to defects in specification or differentiation of parietal, zymogenic and pit cell lineages within primordial buds. Previous studies have shown that ablation of parietal cells results in impaired differentiation of zymogenic cells (Li *et al.*, 1996). Hence, the effect of GATA-4 deficiency on zymogenic cell differentiation may be indirect.

Since gastric unit morphogenesis is not completed until after birth (see Introduction), we extended our analysis to P2. For these experiments *Gata4*$^{-/-}$ ES cells tagged with a *lacZ* expression plasmid (Kuo *et al.*, 1997) were injected into C57Bl/6 *Gata4*$^{+/+}$ blastocysts. Mice were sacrificed at E14.5 and P2. We reasoned that this approach might afford better detection of low percentage glandular gastric epithelium, as it is easier to identify small numbers of blue stained cells on a white background than white cells on a blue background. Consistent with the results obtained from *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimeras, lacZ-tagged ES cells contributed to lung and intestinal epithelia, but not to the glandular epithelium of the stomach of these mice ($n = 5$ at E14.5; $n = 21$ at E15.5; $n = 7$ at P2; data not shown)

Aberrant Shh expression in Gata4*$^{-/-}$ *epithelium. During embryonic development, *Shh* is robustly expressed in proximal (i.e., anterior) stomach epithelium, but only weakly in more distal (i.e., posterior) stomach (Bitgood and McMahon, 1995). Kim *et al.* (2000) have shown that *Shh* expression is attenuated in the distal stomach through activin receptor-mediated signaling. Furthermore, ectopic expression of Shh in the distal stomach epithelium or in pancreatic endoderm of the mouse is associated with aberrant endoderm development (Apelqvist *et al.*, 1997; Kim *et al.*, 2000). Constitutive Shh signaling in *Xenopus* gut mesenchyme results in abnormal epithelial cytodifferentiation (Zhang *et al.*, 2001).

We examined Shh expression in E14.5 *Gata4*$^{-/-}$ ↔ *Gata4*$^{+/+}$ *ROSA26* chimeras. Shh mRNA levels were equivalent in neighboring patches of wild type and *Gata4*$^{-/-}$ epithelium located in the proximal stomach (Figs. 10A and 10B). Proceeding distally, *Shh* expression was augmented in *Gata4*$^{-/-}$ compared to *Gata4*$^{+/+}$ epithelium (Figs. 10C–10F). These findings suggest that GATA-4 may lie downstream of the activin receptor mediated signals that normally attenuate *Shh* expression in the distal stomach.

DISCUSSION

Analysis of chimeric mice generated by introducing *Gata4*$^{-/-}$ ES cells into *Gata4*$^{+/+}$ morulae reveals that GATA-4 deficient cells exhibit an intrinsic defect in gastric epithelial cell differentiation. Within the distal stomach of E14.5 mice, *Gata4*$^{-/-}$ cells express early markers of epithelial differentiation, including GATA-6 and ApoJ. However, by E18.5, *Gata4*$^{-/-}$ cells fail to form primitive gastric units or to support terminal differentiation of the principal gastric epithelial lineages. These effects are cell autonomous; they do not generalize to juxtaposed *Gata4*$^{+/+}$ epithelium. The results of this study, coupled with the lack of a gastric phenotype in mice deficient in GATA-5 (Molkentin *et al.*, 2000) or GATA-6 (Koutsourakis *et al.*, 1999; Morrisey *et al.*, 1998), provide genetic evidence that GATA-4 serves a unique role in regulating cytodifferentiation of definitive endoderm to the stomach's glandular epithelium.

In vitro studies have shown that GATA-4 (or the closely related factors GATA-5 and GATA-6) can bind and *trans*-activate several genes expressed in differentiating and terminally differentiated members of the parietal and zymogenic cell lineages, including the *β-subunit* gene of *H^+/K^+-ATPase* (Mushiake *et al.*, 1994; Nishi *et al.*, 1997a; Tamura *et al.*, 1993) and *pepsinogen* (Sakamoto *et al.*, 1998, 2000). Our results underscore the *in vivo* contributions of GATA-4 to expression of these genes. In addition, *Gata4*$^{-/-}$ distal gastric epithelium exhibits augmented expression of *Shh*, suggesting that this transcription factor plays a direct or indirect role in limiting its expression. Previous studies have shown that downregulation of Shh signaling is re-

quired for gut organogenesis (Apelqvist *et al.*, 1997; Kim *et al.*, 2000; Zhang *et al.*, 2001).

Gene mutations that disrupt the balance between TGF-β/activin and BMP signals affect mouse foregut/stomach development and recapitulate aspects of the *Gata4*$^{-/-}$ phenotype. Studies in chimeric mice have demonstrated that Smad2, which mediates TGFβ-related signaling, is required for formation of gut endoderm (Tremblay *et al.*, 2000). Differentiation and/or migration of definitive endoderm cells is impaired in embryos lacking Furin/SP1, a protease involved in proteolytic maturation of TGF-β, BMP-4, and Nodal precursors (Constam and Robertson, 2000). Mice deficient in the type II activin receptors, ActRIIA and ActRIIB, have disrupted development of the glandular stomach, pancreas, and spleen (Kim *et al.*, 2000). *ActRIIA*$^{+/-}$ *ActRIIB*$^{-/-}$ embryos exhibit ectopic expression of *Shh* and transformation of the distal stomach epithelium from columnar to stratified squamous morphology, a phenotype reminiscent of *Gata4*$^{-/-}$ chimeras. Intriguingly, persistent stimulation of type II activin receptors with supraphysiological levels of activins also disrupts gastric epithelial cell differentiation (Li *et al.*, 1998; Matzuk *et al.*, 1995). Collectively, these similarities among phenotypes provide circumstantial evidence that GATA-4 is involved in the response of gastric epithelium to TGF-β superfamily members. Additional evidence that GATA factors mediate TGF-β superfamily signaling comes from studies with chick (Narita *et al.*, 2000; Sakamoto *et al.*, 2000), zebrafish (Reiter *et al.*, 1999, 2001; Rodaway *et al.*, 1999), and *Xenopus* (Weber *et al.*, 2000) embryos, and from experiments on mouse P19 embryonal carcinoma cells (Monzen *et al.*, 1999).

The activity of GATA binding proteins can be modulated through interactions with other transcription factors, coactivators, and repressors, including factors independently implicated in endoderm development (reviewed in Molkentin, 2000). For example, GATA-4 cooperates with fork head family members, such as HNF3β, to impart genes with the potential to be activated in endodermal tissues (Zaret, 1999). Tetraploid chimera experiments have shown that embryos deficient in HNF3β lack anterior and midgut tissues (Dufort *et al.*, 1998). GATA-4 interacts synergistically with Nkx2.5 to activate an assortment of target genes in the heart (Sepulveda *et al.*, 1998). Intriguingly, Nkx2.5 and other *tinman* family members are expressed in the developing foregut (Biben *et al.*, 1998; Evans *et al.*, 1995; Lee *et al.*, 1996; Oster *et al.*, 1998; Reecy *et al.*, 1997; Sussel *et al.*, 1998). Introduction of a dominant negative Nkx2.5 into chick gut is associated with decreased gland formation in the proventriculus (Smith *et al.*, 2000).

Recent studies have identified genes expressed selectively in human gastroesophageal cancers. *GATA4* is amplified and overexpressed in a subset of gastric and esophageal adenocarcinomas, but not in human lung adenocarcinomas, esophageal squamous cell carcinomas, or Barrett's esophagus, a metaplastic epithelial precursor lesion for esophageal adenocarcinoma (Bai *et al.*, 2000; Lin *et al.*, 2000). These findings suggest that GATA-4 may play a role in the development or maintenance of the malignant glandular phenotype in gastroesophageal adenocarcinomas. The association between human *GATA4* and gastric adenocarcinoma resonates with our studies linking murine *Gata4* to gastric gland differentiation. Of note, enhanced or persistent expression of GATA-4 has been documented in other malignant tumors, including adrenocortical carcinoma (Kiiveri *et al.*, 1999), ovarian/stromal cell tumors (Laitinen *et al.*, 2000), and endodermal sinus tumor (Siltanen *et al.*, 1999). In contrast, benign adrenal or gonadal tumors do not express GATA-4 (Kiiveri *et al.*, 1999; Siltanen *et al.*, 1999). That GATA-4 expression is preserved or enhanced in tissues during malignant transformation underscores the importance of this factor in the regulation of epithelial cell proliferation.

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REFERENCES

Al-azzeh, E., Fegert, P., Blin, N., and Gott, P. (2000). Transcription factor GATA-6 activates expression of gastroprotective trefoil genes TFF1 and TFF2. *Biochim. Biophys. Acta* **1490,** 324–332.

Apelqvist, A., Ahlgren, U., and Edlund, H. (1997). Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. *Curr. Biol.* **7,** 801–804.

Arceci, R. J., King, A.A.J., Simon, M. C., Orkin, S. H., and Wilson, D. B. (1993). Mouse GATA-4: A retinoic acid-inducible GATA-binding transcription factor expressed in endodermal derivatives and heart. *Mol. Cell. Biol.* **13,** 2235–2246.

Aronow, B. J., Lund, S. D., Brown, T. L., Harmony, J. A., and Witte, D. P. (1993). Apolipoprotein J expression at fluid-tissue interfaces: Potential role in barrier cytoprotection. *Proc. Natl. Acad. Sci. USA* **90,** 725–729.

Bai, Y. Q., Akiyama, Y., Nagasaki, H., Yagi, O. K., Kikuchi, Y., Saito, N., Takeshita, K., Iwai, T., and Yuasa, Y. (2000). Distinct expression of CDX2 and GATA4/5, development-related genes, in human gastric cancer cell lines. *Mol. Carcinog.* **28,** 184–188.

Biben, C., Hatzistavrou, T., and Harvey, R. P. (1998). Expression of NK-2 class homeobox gene Nkx2–6 in foregut endoderm and heart. *Mech. Dev.* **73,** 125–127.

Bielinska, M., Narita, N., and Wilson, D. B. (1999). Distinct roles for visceral endoderm during embryonic mouse development. *Int. J. Dev. Biol.* **43,** 183–205.

Bitgood, M. J., and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell–cell interaction in the mouse embryo. *Dev. Biol.* **172,** 126–138.

Bossard, P., and Zaret, K. S. (1998). GATA transcription factors as potentiators of gut endoderm differentiation. *Development* **125,** 4909–4917.

Bossard, P., and Zaret, K. S. (2000). Repressive and restrictive mesodermal interactions with gut endoderm: Possible relation to Meckel's Diverticulum. *Development* **127,** 4915–4923.

Constam, D. B., and Robertson, E. J. (2000). Tissue-specific requirements for the proprotein convertase furin/SPC1 during embryonic turning and heart looping. *Development* **127,** 245–254.

Crispino, J. D., Lodish, M. B., Thurberg, B. L., Litovsky, S. H., Collins, T., Molkentin, J. D., and Orkin, S. H. (2001). Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. *Genes Dev*. **15,** 839–844.

Denson, L. A., McClure, M. H., Bogue, C. W., Karpen, S .J., and Jacobs, H .C. (2000). HNF3beta and GATA-4 transactivate the liver-enriched homeobox gene, Hex. *Gene* **246,** 311–320.

Dufort, D., Schwartz, L., Harpal, K., and Rossant, J. (1998). The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. *Development* **125,** 3015–3025.

Evans, S. M., Yan, W., Murillo, M. P., Ponce, J., and Papaiopulu, N. (1995). *tinman*, a *Drosophila* homeobox gene required for heart and visceral mesoderm specification, may be represented by a family of genes in vertebrates: *XNkx-2.3*, a second vertebrate homologue of *tinman*. *Development* **121,** 3889–3899.

Falk, P., Roth, K. A., and Gordon, J. I. (1994). Lectins are sensitive tools for defining the differentiation programs of mouse gut epithelial cell lineages. *Am. J. Physiol.* **266,** G987–G1003.

Friedrich, G., and Soriano, P. (1991). Promoter traps in embryonic stem cells: A genetic screen to identify and mutate developmental genes in mice. *Genes Dev*. **5,** 1513–1523.

Fukushige, T., Hendzel, M. J., Bazett-Jones, D. P., and McGhee, J. D. (1999). Direct visualization of the elt-2 gut-specific GATA factor binding to a target promoter inside the living *Caenorhabditis elegans* embryo. *Proc. Natl. Acad. Sci. USA* **96,** 11883–11888.

Ghatpande, S., Ghatpande, A., Zile, M., and Evans, T. (2000). Anterior endoderm is sufficient to rescue foregut apoptosis and heart tube morphogenesis in an embryo lacking retinoic acid. *Dev. Biol.* **219,** 59–70.

Grapin-Botton, I., and Melton, D. A. (2000). Endoderm development: From patterning to organogenesis. *Trends Genet*. **16,** 124–130.

Hayashi, K., Yasugi, S., and Mizuno, T. (1988). Pepsinogen gene transcription induced in heterologous epithelial–mesenchymal recombinations of chicken endoderms and glandular stomach mesenchyme. *Development* **103,** 725–731.

Heikinheimo, M., Scandrett, J. M., and Wilson, D. B. (1994). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev. Biol.* **164,** 361–373.

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994). "Manipulating the Mouse Embryo: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C.-N., Simon, M. C., Leiden, J. M., and Parmacek, M. S. (1994). The GATA-4 transcription factor transactivates the cardiac-specific troponin C promoter-enhancer in non-muscle cells. *Mol. Cell. Biol.* **14,** 7517–7526.

Kaestner, K. H., Silberg, D. G., Traber, P. G., and Schutz, G. (1997). The mesenchymal winged helix transcription factor Fkh6 is required for the control of gastrointestinal proliferation and differentiation. *Genes Dev*. **11,** 1583–1595.

Karam, S. M. (1998). Cell lineage relationship in the stomach of normal and genetically manipulated mice. *Braz. J. Med. Biol. Res.* **31,** 271–279.

Karam, S. M., Li, Q., and Gordon, J. I. (1997). Gastric epithelial morphogenesis in normal and transgenic mice. *Am. J. Physiol.* **272,** G1209–G1220.

Ketola, I., Rahman, N., Toppari, J., Bielinska, M., Porter-Tinge, S. B., Tapanainen, J. S., Huhtaniemi, I. T., Wilson, D. B., and Heikinheimo, M. (1999). Expression and regulation of transcription factors GATA-4 and GATA-6 in developing mouse testis. *Endocrinology* **140,** 1470–1480.

Kiiveri, S., Siltanen, S., Rahman, N., Bielinska, M., Lehto, V. P., Huhtaniemi, I. T., Muglia, L. J., Wilson, D. B., and Heikinheimo, M. (1999). Reciprocal changes in the expression of transcription factors GATA-4 and GATA-6 accompany adrenocortical tumorigenesis in mice and humans. *Mol. Med*. **5,** 490–501.

Kim, S. K., Hebrok, M., Li, E., Oh, S. P., Schrewe, H., Harmon, E. B., Lee, J. S., and Melton, D. A. (2000). Activin receptor patterning of foregut organogenesis. *Genes Dev*. **14,** 1866–1871.

Koike, T., and Yasugi, S. (1999). In vitro analysis of mesenchymal influences on the differentiation of stomach epithelial cells of the chicken embryo. *Differentiation* **65**, 13–25.

Kostetskii, I., Jiang, Y., Kostetskaia, E., Yuan, S., Evans, T., and Zile, M. (1999). Retinoid signaling required for normal heart development regulates GATA-4 in a pathway distinct from cardiomyocyte differentiation. *Dev. Biol.* **206**, 206–218.

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R., and Grosveld, F. (1999). The transcription factor GATA6 is essential for early extraembryonic development. *Development* **126,** 723–732.

Kuo, C. T., Morrisey, E. E., Anadappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C., and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev*. **11,** 1048–1060.

Laitinen, M. P., Anttonen, M., Ketola, I., Wilson, D. B., Ritvos, O., Butzow, R., and Heikinheimo, M. (2000). Transcription factors GATA-4 and GATA-6 and a GATA family cofactor, FOG-2, are expressed in human ovary and sex cord-derived ovarian tumors. *J. Clin. Endocrinol. Metab*. **85,** 3476–3483.

Lee, K.-H., Xu, Q., and Breitbart, R. E. (1996). A new tinman-related gene, nkx2.7, anticipates the expression of nkx2.5 and nkx2.3 in zebrafish heart and pharyngeal endoderm. *Dev. Biol.* **180,** 722–731.

Li, Q., Karam, S. M., Coerver, K. A., Matzuk, M. M., and Gordon, J. I. (1998). Stimulation of activin receptor II signaling pathways inhibits differentiation of multiple gastric epithelial lineages. *Mol. Endocrinol.* **12,** 181–192.

Li, Q., Karam, S. M., and Gordon, J. I. (1995). Simian virus 40 T antigen-induced amplification of pre-parietal cells in transgenic mice. Effects on other gastric epithelial cell lineages and evidence for a p53-independent apoptotic mechanism that operates in a committed progenitor. *J. Biol. Chem*. **270,** 15777–15788.

Li, Q., Karam, S. M., and Gordon, J. I. (1996). Diphtheria toxin-mediated ablation of parietal cells in the stomach of transgenic mice. *J. Biol. Chem*. **271,** 3671–3676.

Lin, L., Aggarwal, S., Glover, T. W., Orringer, M. B., Hanash, S., and Beer, D. G. (2000). A minimal critical region of the 8p22–23 amplicon in esophageal adenocarcinomas defined using sequence tagged site-amplification mapping and quantitative polymerase chain reaction includes the GATA-4 gene. *Cancer Res.* **60,** 1341–1347.

Lorenz, R. G., and Gordon, J. I. (1993). Use of transgenic mice to study regulation of gene expression in the parietal cell lineage of gastric units. *J. Biol. Chem.* **268,** 26559–26570.

Matzuk, M. M., Kumar, T. R., and Bradley, A. (1995). Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature.* **374,** 356–600.

Molkentin, J. D. (2000). The zinc finger-containing transcription factors GATA-4, -5, and -6: Ubiquitously expressed regulators of tissue-specific gene expression. *J. Biol. Chem.* **275,** 38949–38952.

Molkentin, J. D., Lin, Q., Duncan, S. A., and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11,** 1061–1072.

Molkentin, J. D., Tymitz, K. M., Richardson, J. A., and Olson, E. N. (2000). Abnormalities of the genitourinary tract in female mice lacking GATA5. *Mol. Cell. Biol.* **20,** 5256–5260.

Monzen, K., Shiojima, I., Hiroi, Y., Kudoh, S., Oka, T., Takimoto, E., Hayashi, D., Hosoda, T., Habara-Ohkubo, A., Nakaoka, T., Fujita, T., Yazaki, Y., and Komuro, I. (1999). Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase kinase kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4. *Mol. Cell. Biol.* **19,** 7096–7105.

Morrisey, E. E., Ip, H. S., Lu, M. M., and Parmacek, M. S. (1996). GATA-6: A zinc finger transcription factor that is expressed in multiple cell lineages derived from the lateral mesoderm. *Dev. Biol.* **177,** 309–322.

Morrisey, E. E., Musco, S., Chen, M. Y., Lu, M. M., Leiden, J. M., and Parmacek, M. S. (2000). The gene encoding the mitogen-responsive phosphoprotein Dab2 is differentially-regulated by GATA-6 and GATA-4 in the visceral endoderm. *J. Biol. Chem.* **275,** 19949–19954.

Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S., and Parmacek, M. S. (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12,** 3579–3590.

Mushiake, S., Etani, Y., Shimada, S., Tokyama, M., Haseke, M., Futai, M., and Maeda, M. (1994). Genes for members of the GATA-binding protein family (GATA-GT1 and GATA-GT2) together with H^+/K^+-ATPase are specifically transcribed in gastric parietal cells. *FEBS Lett.* **340,** 117–120.

Narita, N., Bielinska, M., and Wilson, D. B. (1997a). Cardiomyocyte differentiation by GATA-4 deficient embryonic stem cells. *Development* **122,** 3755–3764.

Narita, N., Bielinska, M., and Wilson, D. B. (1997b). Wild type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. *Dev. Biol.* **189,** 270–274.

Narita, N., Heikinheimo, M., Bielinska, M., White, R. A., and Wilson, D. B. (1996). The gene for transcription factor GATA-6 resides on mouse chromosome 18 and is expressed in myocardium and vascular smooth muscle. *Genomics* **36,** 345–348.

Narita, T., Saitoh, K., Kameda, T., Kuroiwa, A., Mizutani, M., Koike, C., Iba, H., and Yasugi, S. (2000). BMPs are necessary for stomach gland formation in the chicken embryo: A study using virally induced BMP-2 and Noggin expression. *Development* **127,** 981–988.

Nishi, T., Kubo, K., Hasebe, M., Maeda, M., and Futai, M. (1997). Transcriptional activation of H^+/K^+-ATPase genes by gastric GATA binding proteins. *J. Biochem.* **121,** 922–929.

Nomura, S., Esumi, H., Job, C., and Tan, S. S. (1998). Lineage and clonal development of gastric glands. *Dev. Biol.* **204,** 124–135.

Oster, A., Jensen, J., Edlund, H., and Larsson, L. I. (1998). Homeobox gene product Nkx 6.1 immunoreactivity in nuclei of endocrine cells of rat and mouse stomach. *J. Histochem. Cytochem.* **46,** 717–721.

Papaioannou, V. E., and Johnson, R. (1993). Production of chimeras and genetically defined offspring from targeted ES cells. In "Gene Targeting: A Practical Approach" (A. L. Joyner, Ed.), pp. 107–146. IRL Press, Oxford.

Paré, J. F., Roy, S., Galarneau, L., and Belanger, L. (2001). The mouse fetoprotein transcription factor (FTF) gene promoter is regulated by three GATA elements with tandem E-box and Nkx motifs, and FTF in turn activates the HNF3B, HNF4a and HNF1a gene promoters. *J. Biol. Chem.* **276,** 13136–13144.

Ramalho-Santos, M., Melton, D. A., and McMahon, A. P. (2000). Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* **127,** 2763–2772.

Reecy, J. M., Yamada, M., Cummings, K., Sosic, D., Chen, C. Y., Eichele, G., Olson, E. N., and Schwartz, R. J. (1997). Chicken Nkx-2.8: A novel homeobox gene expressed in early heart progenitor cells and pharyngeal pouch-2 and -3 endoderm. *Dev. Biol.* **295,** 3107–3111.

Rehorn, K. P., Thelen, H., Michelson, A. M., and Reuter, R. (1996). A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila. Development* **122,** 4023–4031.

Reiter, J. F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N., and Stainier, D. Y. (1999). Gata5 is required for the development of the heart and endoderm in zebrafish. *Genes Dev.* **13,** 2983–2995.

Reiter, J. F., Kikuchi, Y., and Stainier, D. Y. (2001). Multiple roles for Gata5 in zebrafish endoderm formation. *Development* **128,** 125–135.

Rodaway, A., Takeda, H., Koshida, S., Broadbent, J., Price, B., Smith, J. C., Patient, R., and Holder, N. (1999). Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-beta family signals and discrimination of mesoderm and endoderm by FGF. *Development* **126,** 3067–3078.

Sakamoto, N., Fukuda, K., Watanuki, K., Sakai, D., Komano, T., Scotting, P. J., and Yasugi, S. (2000). Role for cGATA-5 in transcriptional regulation of the embryonic chicken pepsinogen gene by epithelial–mesenchymal interactions in the developing chicken stomach. *Dev. Biol.* **223,** 103–113.

Sakamoto, N., Saiga, H., and Yasugi, S. (1998). Analysis of temporal expression pattern and cis-regulatory sequences of chicken pepsinogen A and C. *Biochem. Biophys. Res. Commun.* **250,** 420–424.

Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., and Schwartz, R .J. (1998). GATA-4 and nkx-2.5 coactivate nkx-2 DNA binding targets: Role for regulating early cardiac gene expression. *Mol. Cell. Biol.* **18,** 3405–3415.

Shoichet, S. A., Malik, T. H., Rothman, J. H., and Shivdasani, R. A. (2000). Action of the *Caenorhabditis elegans* GATA factor END-1 in Xenopus suggests that similar mechanisms initiate endoderm development in ecdysozoa and vertebrates. *Proc. Natl. Acad. Sci. USA* **97,** 4076–4081.

Siltanen, S., Anttonen, M., Heikkila, P., Narita, N., Laitinen, M., Ritvos, O., Wilson, D. B., and Heikinheimo, M. (1999). Transcription factor GATA-4 is expressed in pediatric yolk sac tumors. *Am. J. Pathol.* **155,** 1823–1829.

Smith, D. M., Nielsen, C., Tabin, C. J., and Roberts, D. J. (2000). Roles of BMP signaling and Nkx2.5 in patterning at the chick midgut-foregut boundary. *Development* **127,** 3671–3681.

Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C. A., Narita, N., Saffitz, J. E., Simon, M. C., Leiden, J. M., and Wilson, D. B. (1995). Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. *Development* **121,** 3877–3888.

Sukegawa, A., Narita, T., Kameda, T., Saitoh, K., Nohno, T., Iba, H., Yasugi, S., and Fukuda, K. (2000). The concentric structure of the developing gut is regulated by Sonic hedgehog derived from endodermal epithelium. *Development* **127,** 1971–1980.

Sussel, L., Kalamaras, J., Hartigan-O'Connor, D. J., Meneses, J J., Pedersen, R. A., Rubenstein, J.L., and German, M. S. (1998). Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development* **125,** 2213–2221.

Syder, A. J., Guruge, J. L., Li, Q., Hu, Y., Oleksiewicz, C. M., Lorenz, R. G., Karam, S. M., Falk, P. G., and Gordon, J. I. (1999). Helicobacter pylori attaches to NeuAc alpha 2,3Gal beta 1,4 glycoconjugates produced in the stomach of transgenic mice lacking parietal cells. *Mol. Cell* **3,** 263–274.

Tabata, H., and Yasugi, S. (1998). Tissue interaction regulates expression of a spasmolytic polypeptide gene in chicken stomach epithelium. *Dev. Growth Differ.* **40,** 519–526.

Tamura, S., Wang, X.-H., Maeda, M., and Futai, M. (1993). Gastric DNA-binding proteins recognize upstream sequence motifs of parietal cell-specific genes. *Proc. Natl. Acad. Sci. USA* **90,** 10876–10880.

Tremblay, K. D., Hoodless, P. A., Bikoff, E. K., and Robertson, E. J. (2000). Formation of the definitive endoderm in mouse is a Smad2-dependent process. *Development* **127,** 3079–3090.

Viger, R. S., Mertineit, C., Trasler, J. M., and Nemer, M. (1998). Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Müllerian inhibiting substance promoter. *Development* **125,** 2665–2755.

Weber, H., Symes, C. E., Walmsley, M. E., Rodaway, A. R., and Patient, R. K. (2000). A role for GATA5 in Xenopus endoderm specification. *Development* **127,** 4345–4360.

Wells, J. M., and Melton, D. A. (1999). Vertebrate endoderm development. *Annu. Rev. Cell Dev. Biol.* **15,** 393–410.

Yasuo, H., and Lemaire, P. (1999). A two-step model for the fate determination of presumptive endodermal blastomeres in *Xenopus* embryos. *Curr. Biol.* **9**, 869–879.

Zaret, K. (1999). Developmental competence of the gut endoderm: Genetic potentiation by GATA and HNF3/fork head proteins. *Dev. Biol.* **209,** 1–10.

Zhang, J., Rosenthal, A., de Sauvage, F. J., and Shivdasani, R. A. (2001). Downregulation of Hedgehog signaling is required for organogenesis of the small intestine in *Xenopus. Dev. Biol.* **229**, 188–202.

Zhu, H., Hill, R., Heid, P. J., Fukuyama, M., Sugimoto, A., Priess, J. R., and Rothman, J. H. (1997). *end-1* encodes an apparent GATA factor that specifies the endoderm precursor in *Caenorhabditis elegans* embryos. *Genes Dev.* **11**, 2883–2896.

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